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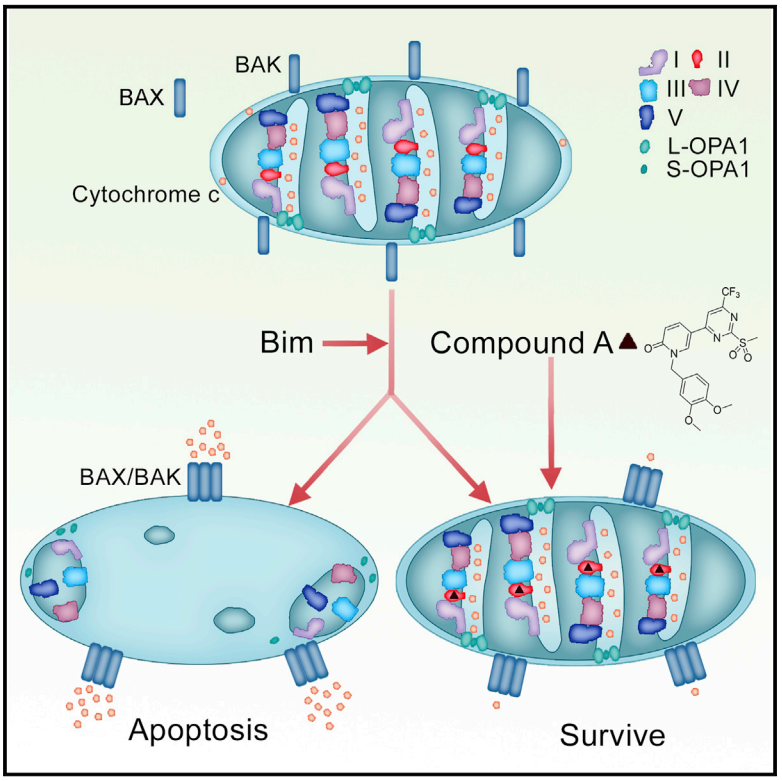
Molecular Cell

A Small Molecule That Protects the Integrity of the Electron Transfer Chain Blocks the Mitochondrial Apoptotic Pathway

Graphical Abstract



Highlights

- The mitochondrial electron transfer chain (ETC) is disrupted during Bim-induced apoptosis
- A small molecule protects the mitochondrial ETC and prevents Bim-induced apoptosis
- A small molecule protects the ETC during apoptosis by targeting SDHB of complex II
- This small molecule prevents dopaminergic neuronal death in a Parkinson's disease model

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In Brief

The mitochondrial electron transfer chain (ETC) is cells' main energy provider and a source of apoptosis signaling when cytochrome *c* is released to the cytosol. Jiang et al. described a small molecule that binds to SDHB of complex II of the ETC and preserves the integrity of the ETC during apoptosis.

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A Small Molecule That Protects the Integrity of the Electron Transfer Chain Blocks the Mitochondrial Apoptotic Pathway

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SUMMARY

In response to apoptotic stimuli, mitochondria in mammalian cells release cytochrome *c* and other apoptogenic proteins, leading to the subsequent activation of caspases and apoptotic cell death. This process is promoted by the pro-apoptotic members of the Bcl-2 family of proteins, such as Bim and Bax, which, respectively, initiate and execute cytochrome *c* release from the mitochondria. Here we report the discovery of a small molecule that efficiently blocks Bim-induced apoptosis after Bax is activated on the mitochondria. The cellular target of this small molecule was identified to be the succinate dehydrogenase subunit B (SDHB) protein of complex II of the mitochondrial electron transfer chain (ETC). The molecule protects the integrity of the ETC and allows treated cells to continue to proliferate after apoptosis induction. Moreover, this molecule blocked dopaminergic neuron death and reversed Parkinson-like behavior in a rat model of Parkinson's disease.

INTRODUCTION

In mammalian cells, upon apoptosis induction, several apoptogenic proteins that are normally located in the intermembrane space of mitochondria overcome the tightly packed cristae of the inner membrane and the physical barrier of the outer membrane to reach the cytosol (reviewed by Danial and Korsmeyer, 2004). The best known such protein is cytochrome *c*, which binds to its cytosolic partner Apaf-1 to form the apoptosome, a heptamer of the Apaf-1/cytochrome *c* complex. The apoptosome recruits and activates procaspase-9 to initiate an apoptotic protease cascade leading to cell death (Li et al., 1997; Zhou et al., 2015).

The Bcl-2 family of proteins controls the release of apoptogenic proteins from mitochondria. The Bax and Bak proteins of the Bcl-2 family function as the gatekeepers for the cytochrome *c* release process (Wei et al., 2001). These two proteins are dormant in live cells and are activated by the sentinel members of the family known as the BH3-only proteins. In response to apoptotic stimuli, BH3-only proteins such as Bim and Bid elevate their levels in cells either through transcription or by transitioning from an inactive to an active state through post-translational modifications (reviewed by Czabotar et al., 2014). Both Bax/Bak and BH3-only family proteins can be sequestered by binding to the anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-X_L (reviewed by Czabotar et al., 2014). Upregulation of the level of Bcl-2 is often seen in various forms of human B cell malignancy. A chemical inhibitor named Venclexta that is specifically designed to prevent Bcl-2 from interacting with pro-apoptotic members of the Bcl-2 family has recently been approved by the Food and Drug Administration for the treatment of patients with chronic lymphocytic leukemia (CLL) who have a chromosomal abnormality called 17p deletion.

Our understanding of the molecular mechanism through which activated Bax and Bak cause the release of cytochrome *c* from mitochondria remains incomplete. The activated Bax and Bak proteins manifest as higher-order protein complexes and have been observed on mitochondria in cells undergoing apoptosis (Eskes et al., 2000; Wei et al., 2001; Antonsson et al., 2001; Nechushtan et al., 2001; Annis et al., 2005). It has been proposed that these complexes function as protein pores or channels that allow proteins such as cytochrome *c* to pass through (Korsmeyer et al., 2000; Dejean et al., 2005; Martinez-Caballero et al., 2009). In vitro experiments utilizing cardiolipin-containing, liposome-wrapped cytochrome *c* have shown that addition of purified Bax, together with activated Bid, was sufficient to release the contents of liposomes (Kuwana et al., 2002; Lovell et al., 2008; Lucken-Ardjomande et al., 2008). However, this proposed model discounted the fact that the majority of cytochrome *c* in mitochondria is tightly packed together with other electron transfer chain components in the inner membrane, which folds into the characteristic structures of cristae. The folded cristae require remodeling to loosen the tight packing to allow the intermembrane space proteins stacked in between the cristae to gain access to the outer membrane (Scorrano et al., 2002). The necks of the cristae that face the outer membrane are sealed by protein complexes consisting of different spliced forms of OPA1, a dynamin-related GTPase that is required for mitochondrial

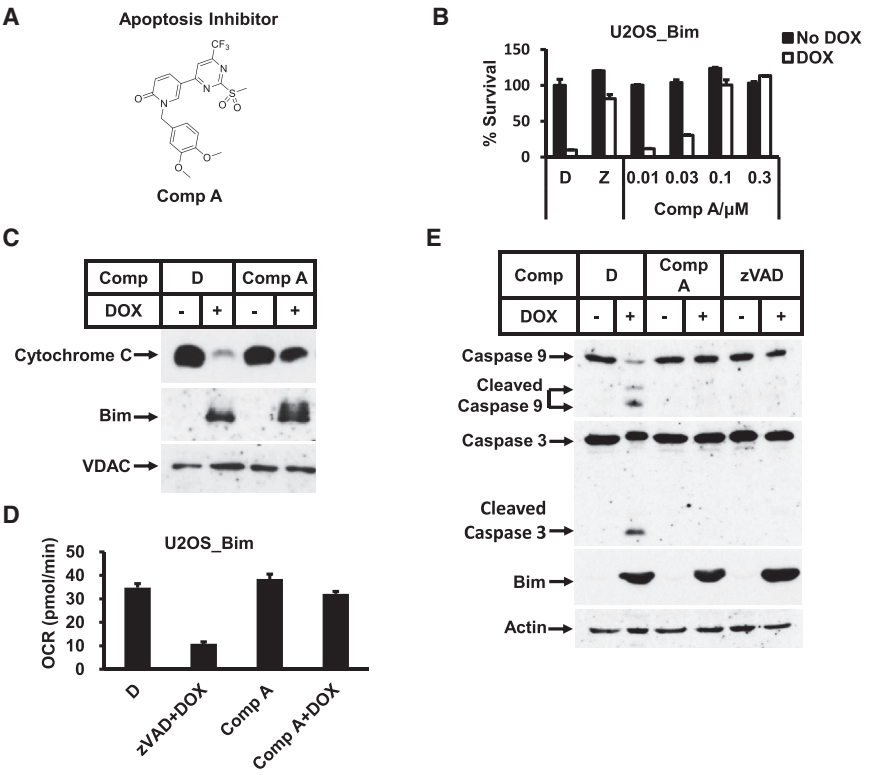

Figure 1. Small-Molecule Compound A Blocked Cytochrome *c* Release during Apoptosis

(A) Chemical structure of compound A (Comp A) (1-(3,4-dimethoxybenzyl)-5-(2-(methylsulfonyl)-6-(trifluoromethyl)pyrimidin-4-yl)pyridin-2(1H)-one).

(B) U2OS_Bim cells were treated with DMSO (D), 20 μM zVAD (Z), or the indicated increasing concentrations of compound A for 1 hr and were then treated with or without DOX for 24 hr. Cell viability was determined using a Cell Titer-Glo kit. Data are represented as the mean ± SD of duplicates. The same concentration of zVAD was used in all experiments.

(C) U2OS_Bim cells were treated with DMSO or compound A (0.1 μM) for 1 hr and were then treated with or without DOX for 8 hr. P15 fractions of the cells were analyzed by western blotting using anti-cytochrome *c*, anti-Bim, and anti-VDAC antibodies. The same concentration of compound A was used in the following experiments unless otherwise stated.

(D) U2OS_Bim cells were treated with DMSO, zVAD, or compound A for 1 hr and were then treated with or without DOX for 12 hr. Oxygen consumption rates of the cells were examined. Data are represented as the mean ± SD of duplicates.

(E) U2OS_Bim cells were treated with DMSO, compound A, or zVAD for 1 hr and were then treated with or without DOX for 8 hr. Whole-cell extracts were analyzed by western blotting using anti-caspase 9, anti-caspase 3, anti-Bim, and anti-β-Actin-HRP antibodies.

fusion; mutations of OPA1 are known to cause optical nerve atrophy in humans (Delettre et al., 2000; Alexander et al., 2000). The cleavage of the long form of OPA1 results in the disassembly of the OPA-1-containing complexes, leading to opening up of the cristae (Olichon et al., 2003; Frezza et al., 2006; Yamaguchi et al., 2008).

In a previous study, we used the doxycycline-inducible expression of Bim or tBid to confirm that OPA1 cleavage by the mitochondrial inner membrane-embedded protease OMA1 is required for cytochrome *c* release from mitochondria after Bax/Bak activation (Jiang et al., 2014). How Bax and Bak activate OMA1 remains unknown.

Taking advantage of this highly specific and precisely controlled apoptosis system, we screened a chemical library of ~200,000 compounds for small molecules that could block the release of cytochrome *c* from the mitochondria after Bim induction. One of the hits from the screen was further characterized by medicinal chemical efforts to define its structure-activity relationship (SAR) and to improve its efficacy. The most efficacious of the resulting compounds was used to identify the cellular target through which the compound exerts its anti-apoptosis function. The target turned out to be the succinate dehydrogenase subunit B (SDHB) protein, a component of complex II of the electron transfer chain (ETC). Characterization of the compound and its interacting target SDHB revealed that the disruption of the integrity of the ETC might be the "point of no return" for apoptosis execution.

RESULTS

A Small Molecule Blocks Cytochrome *c* Release

The human osteosarcoma-U2OS-derived cell line U2OS_Bim underwent robust apoptosis when Bim expression was induced upon the addition of doxycycline (DOX) to the culture medium. Within a few hours, more than 90% of the cells had died (Jiang et al., 2014). A chemical library of more than 200,000 compounds was used to screen for inhibitors that blocked this form of apoptosis. One of the hits from this screen was further subjected to medicinal chemical modification to yield the most potent compound: 1-(3,4-dimethoxybenzyl)-5-(2-(methylsulfonyl)-6-(trifluoromethyl)pyrimidin-4-yl)pyridin-2(1H), hereafter named compound A (Figure 1A). This compound inhibits Bim-induced apoptosis in a dose-dependent manner, with a half-maximal inhibitory concentration (IC_{50}) around 50 nM (Figure 1B). At the 100 nM concentration, compound A blocked the release of the majority of cytochrome *c* from mitochondria, whereas Bim accumulation on the mitochondria was not affected (Figure 1C). Additionally, a decrease in the rate of respiration upon Bim induction, as measured by oxygen consumption, was blocked by compound A. In contrast, treatment with the pan-caspase inhibitor z-VAD-FMK (zVAD) did not prevent the decrease of respiration even though it completely blocked caspase-9 and caspase-3 activation (Figures 1D and 1E). Consistently, no caspase-9 or caspase-3 activation was observed when compound A was present, despite similar amounts of Bim on the mitochondria (Figure 1E). In addition to

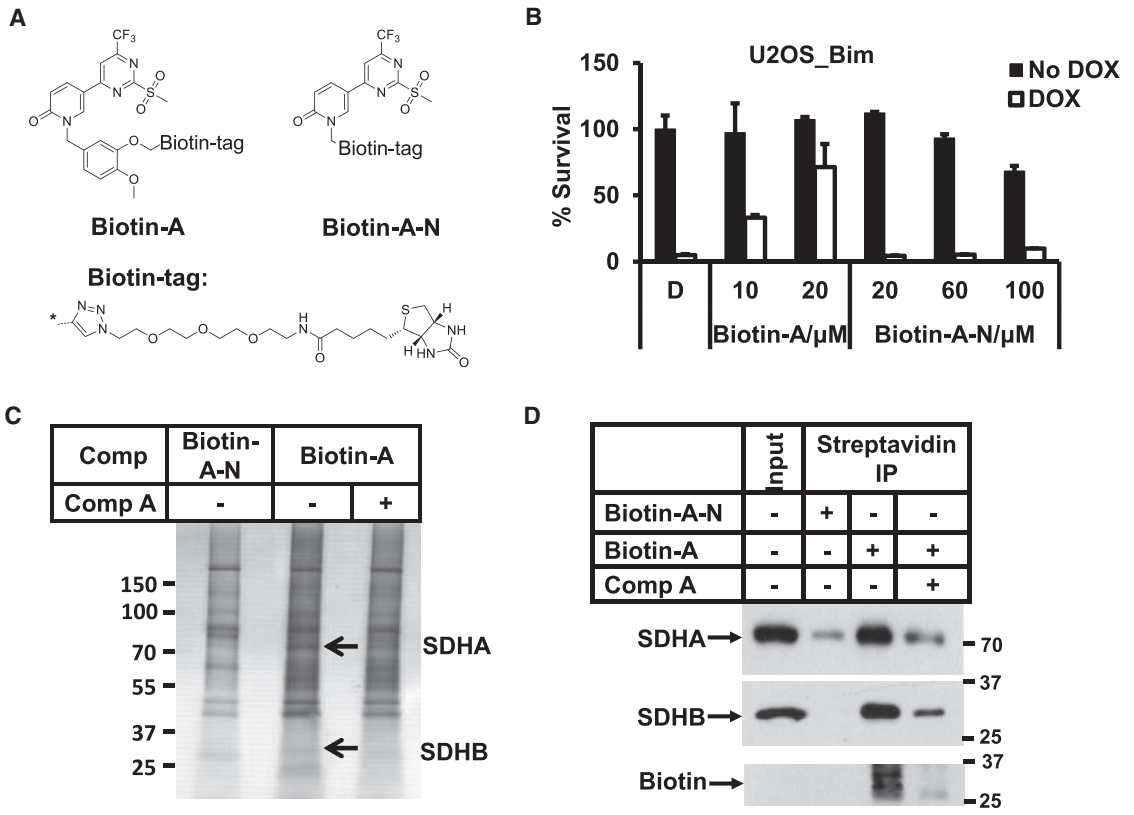

Figure 2. Compound A Binds to SDHB

(A) Chemical structures of Biotin-A (biotin-labeled compound A) and Biotin-A-N (a biotin-labeled inactive compound A analog).

(B) U2OS_Bim cells were treated with DMSO or the indicated increasing concentrations of Biotin-A and Biotin-A-N for 1 hr and were then treated with or without DOX for 24 hr. Cell survival was determined using a Cell Titer-Glo kit. Data are represented as the mean ± SD of duplicates.

(C and D) Lysates of the P15 fractions of U2OS_Bim cells were incubated with or without compound A (1 μM) for 2 hr and were then immunoprecipitated (IP) with Biotin-A (20 μM) or Biotin-A-N (20 μM)-coated streptavidin agarose beads as described in the Experimental Procedures. Silver staining (C) or western blotting with anti-SDHA, anti-SDHB, and anti-streptavidin-HRP antibodies (D) was performed. The same concentration of Biotin-A was used in the following experiments.

cell vitality, cytochrome *c* release, and caspase-9/3 activation, we also checked whether compound A had any effect on mitochondrial crista remodeling that might occur after apoptosis induction. Indeed, addition of DOX to U2OS-Bim cells caused more than 80% of the mitochondria to lose their crista structure, and treatment with compound A preserved the majority of crista structures (Figures S1A and S1B).

In addition to Bim-mediated apoptosis, we also tested the effect of compound A in U2OS cells treated with the chemotherapy drug etoposide and in a human glioblastoma cell line, T98G, that had been irradiated with ultraviolet light. We found that compound A was able to block caspase-9 activation in cell lines treated with these diverse kinds of apoptotic stimuli that activate apoptosis through the mitochondrial pathway, although it should be noted that its efficacy was about 10- to 30-fold lower here than in Bim-induced apoptosis (Figures S1C and S1D). In contrast, compound A at a 10-μM concentration (100-fold higher than the concentration to completely block Bim-induced apoptosis) did not block apoptosis in HeLa cells induced by the extrinsic apoptotic pathway stimulus tumor necrosis factor α (TNF-α) plus a Smac mimetic, nor did it block necrotic cell death in U2OS cells induced by tert-butyl-peroxide (tBH) (Figures S1E and S1F). These results indicated that compound A blocks apoptosis at a step that occurs prior to crista remodeling and cytochrome *c* release but after Bim is localized on mitochondria.

Identification of Succinate Dehydrogenase Subunit B as the Cellular Target of Compound A

To identify the cellular target through which compound A exerts its cell-protective activity, we synthesized a derivative of compound A with a biotin tag linked to its benzyl ring (Figure 2A). The biotin-labeled compound A was less potent than the unmodified compound A; it took a 20-μM concentration to save ~80% of cells (Figure 2B). A similarly biotin-tagged molecule that further lacked the benzyl ring of compound A (referred to as Biotin-A-N) showed no activity; this was used as a negative control in subsequent experiments (Figures 2A and 2B).

We isolated the mitochondrion-enriched fraction from U2OS_Bim cells and lysed this material in buffer containing the detergent Triton X-100. The solubilized fraction was then incubated with streptavidin beads that had been coated with the two biotin-labeled compounds. The proteins associated with the

beads were analyzed by SDS-PAGE and silver staining. As indicated in Figure 2C, two proteins, one at the 70-kDa marker and the other above the 25-kDa marker, were specifically precipitated with compound A-biotin but not with the inactive Biotin-A-N. The two proteins were also specifically competed out when the unmodified compound A was mixed with the lysates before they were incubated with biotin-labeled compound A streptavidin beads, whereas the other protein bands still bound to the beads, indicating that two protein bands near the 70-kDa marker and above the 25-kDa marker were the only proteins specifically bound to compound A.

The two proteins were subsequently identified by mass spectrometry as succinate dehydrogenase subunit A (70 kDa) and subunit B (32 kDa), two of the known components of complex II of the mitochondrial ETC. The identity of these two proteins was further verified by western blotting analysis of the biotin-streptavidin bead pull-down protein samples using antibodies against each of the two proteins (Figure 2D). The western blotting results showed that the biotin-labeled variant of compound A pulled down both SDHA and SDHB, whereas the inactive Biotin-A-N-streptavidin beads only had a background level of SDHA associated with it (Figure 2D, second and third lanes). Consistent with the silver staining results presented in Figure 2C, the majority of the population of these two proteins associated with the biotin-compound A-streptavidin beads were competed out when the unmodified compound A was present (Figure 2D, last two lanes).

Compound A Covalently Binds to Cysteine 243 of SDHB

The structural features of compound A suggested its likely capacity to covalently modify target proteins, as its methylsulfonyl moiety can serve as a leaving group when attacked by a nucleophile such as cysteine (Figure S2A). Indeed, when we performed a western blot analysis of the proteins associated with biotin-labeled compound A-streptavidin beads with an antibody against biotin, we detected a signal that migrated to the same position as SDHB on SDS-PAGE gels, indicating that SDHB might be the direct target of compound A. Furthermore, it is clear that the covalent bonding between the biotin-modified compound A and SDHB was strong enough to withstand the SDS-PAGE and western blotting procedures (Figure 2D, bottom). The pulled-down SDHA did not contain any biotin signal and thus must have been pulled down as an associated partner of SDHB.

When the protein band of SDHB from the silver staining was digested and the peptides were analyzed by mass spectrometry, a peptide with the amino acid residue sequence of CHTIMNCTR was identified to contain a biotin-labeled compound A modification (C39H46F3N9O8S, molecular weight [MW] 857.9) (Figure 3A). The b-type (N-terminal) and y-type (C-terminal) fragments of the peptide data are also shown in Figure 3A. The mass shift occurred in all of the b-type fragments; this modification begins at the b2 residue. Therefore, the modification site must be the first cysteine of this peptide, which corresponds to cysteine 243 of SDHB.

Another clear indication that compound A covalently binds to its target was that, after U2OS_Bim cells were treated with compound A and then washed with fresh medium, the cell protection activity remained strong for at least 24 hr following the wash (Figure S2B).

To verify the specificity of cysteine 243 of SDHB in binding compound A, we ectopically expressed wild-type SDHB and a C243A mutant version of SDHB in HeLa cells. Both of these recombinant proteins were tagged with FLAG tags. As shown in Figure 3B, both proteins were expressed at levels very similar to that of endogenous SDHB, and both proteins migrated higher on the SDS-PAGE gel because of the FLAG tag. When the mitochondrion-enriched fractions were prepared and subjected pull-down with biotin-labeled compound A/streptavidin beads, the wild-type SDHB was pulled down, whereas very little C243A mutant protein was pulled down, as measured by western blotting using both an anti-SDHB antibody and an anti-FLAG antibody (Figure 3B, two top panels).

It was thus clear that cysteine 243 is required for the binding of compound A to SDHB. To test whether the cell protection activity of compound A did indeed occur through the binding of SDHB, we employed CRISPR-CAS9 technology to generate a specific TG-to-GC mutation in exon 7 of the SDHB gene that resulted in a cysteine 243-to-alanine (C243A) mutation in the protein (Figure S3; Figure 3C). When analyzed by western blotting using extracts from cells grown up from two independently isolated knockin cell clones, the mutant protein was found to express at normal levels relative to the wild-type (Figure 3D). Moreover, the rate of respiration in these cells was similar to the wild-type level (Figure 3E). The cells carrying the C243A knockin mutation died normally when DOX was added to the culture medium. In contrast to wild-type cells, compound A protection was largely lost in these cells, confirming that the cell protection activity of compound A indeed occurs through its bonding with cysteine 243 of SDHB (Figure 3F).

Compound A Prevents the Disruption of Complex II of the ETC but Not Bax Association with Mitochondria and Bax Oligomerization

Given that compound A targets a component of complex II of the ETC, we analyzed complex II at different time points after Bim induction using blue native gel electrophoresis followed by western blotting using antibodies against SDHA. As shown in Figure 4A, top, complex II of the ETC was disrupted 8 hr after Bim expression was induced. Interestingly, Bim protein was observed in the mitochondrion-enriched fraction 2 hr after induction, and Bax was present in this fraction after 4 hr, suggesting that Bim induction, Bax translocation to mitochondria, and complex II disruption are sequential events. The levels of SDHA and SDHB were constant throughout the time course. Surprisingly, the SDHC protein, the complex II subunit that links the SDHA/SDHB subunits to SDHC/SDHD on the mitochondrial inner membrane (Sun et al., 2005), was degraded 8 hr after Bim induction. The time of SDHC degradation was coincident with the disruption of complex II. The presence of compound A preserved the integrity of complex II, prevented the degradation of SDHC, and prevented the activation of OMA1. Compound A did not, however, interrupt the association of Bim and Bax with the mitochondria and did not prevent oligomerization of Bax (Figures 4B and 4C). The amounts of Bim associated with the mitochondria were similar in the presence or absence of compound A, but

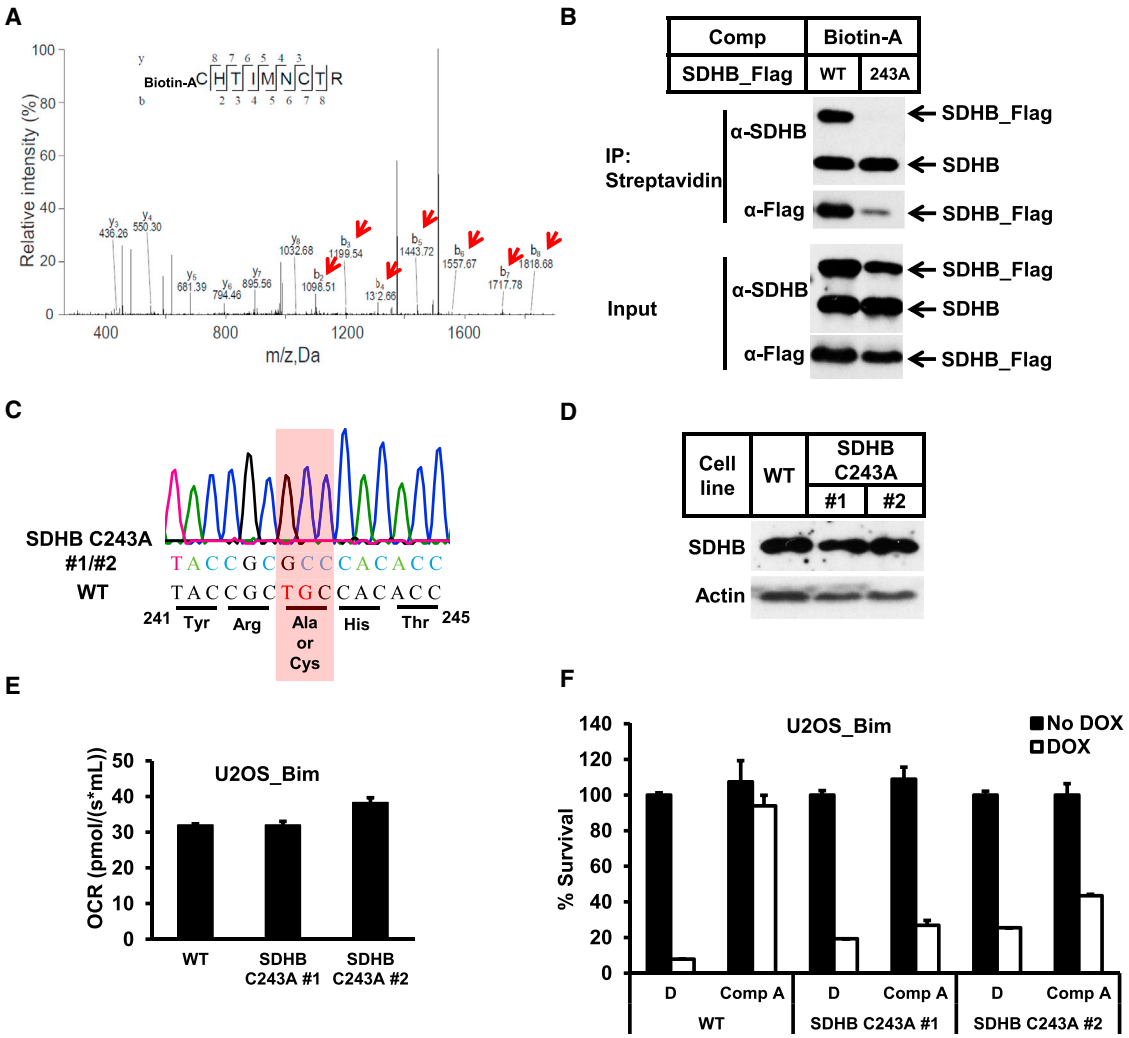

Figure 3. Compound A Covalently Binds to Cysteine 243 of SDHB

(A) Mass spectrum of the Biotin-A binding peptide of SDHB. A Biotin-A modification (C39H46F3N9O8S, MW 857.9) was identified in this CHTIMNCTR peptide at the C243 residue. The y-type (C-term) and b-type (N-term) product ions are shown in the spectrum. Note that there was a mass shift in all b-type ions (denoted by red arrows).

(B) HeLa cells were transfected with FLAG-tagged wild-type (WT) SDHB or C243A mutant of SDHB for 24 hr. Lysates of the P15 fractions of the cells were immunoprecipitated with Biotin-A-coated streptavidin agarose beads, followed by western blotting using anti-SDHB and anti-FLAG-HRP antibodies.

(C) The U2OS_Bim SDHB C243A knockin cell line was generated as described in the Experimental Procedures. Sequencing results for the region surrounding the codon for the 243rd amino acid of SDHB in SDHB knockin clones 1 and 2 are shown and are aligned with the corresponding sequence in the wild-type cell line. The corresponding amino acids are shown under the DNA sequence. The mutated site (243rd amino acid) is shaded in red.

(D) Whole cell lysates of U2OS_Bim wild-type and SDHB C243A knockin clone 1 and 2 cells were analyzed by western blotting using anti-SDHB and anti-β-Actin-HRP antibodies.

(E) The respiration rates of U2OS_Bim wild-type and SDHB C243A knockin clone 1 and 2 cells were measured as described in the Experimental Procedures. Data are represented as the mean ± SD of duplicates.

(F) U2OS_Bim wild-type and SDHB C243A knockin cell line clones 1 and 2 were treated with DMSO or compound A for 1 hr, and cells were then treated with or without DOX for 24 hr. Cell viability was determined using a Cell Titer-Glo kit. Data are represented as the mean ± SD of duplicates.

the amount of Bax appeared to be reduced in the presence of compound A. It is likely that the translocation of Bax to mitochondria and its oligomerization might be accelerated by the damage to the ETC. In addition to complex II of the ETC, the complex II-containing ETC supercomplex was also preserved in the presence of compound A (Acín-Pérez et al., 2008; Cogliati et al., 2013; Figure S4A).

To confirm that the disruption of complex II of the ETC is due to Bax/Bak, we repeated these experiments using a derived U2OS_Bim cell line in which both Bax and Bak were stably knocked down by small hairpin RNA (shRNA) (Figure S4B). The addition of DOX did not cause any disruption of ETC complex II in these cells, and the degradation of SDHC was also precluded (Figure S4C), indicating that both the degradation of

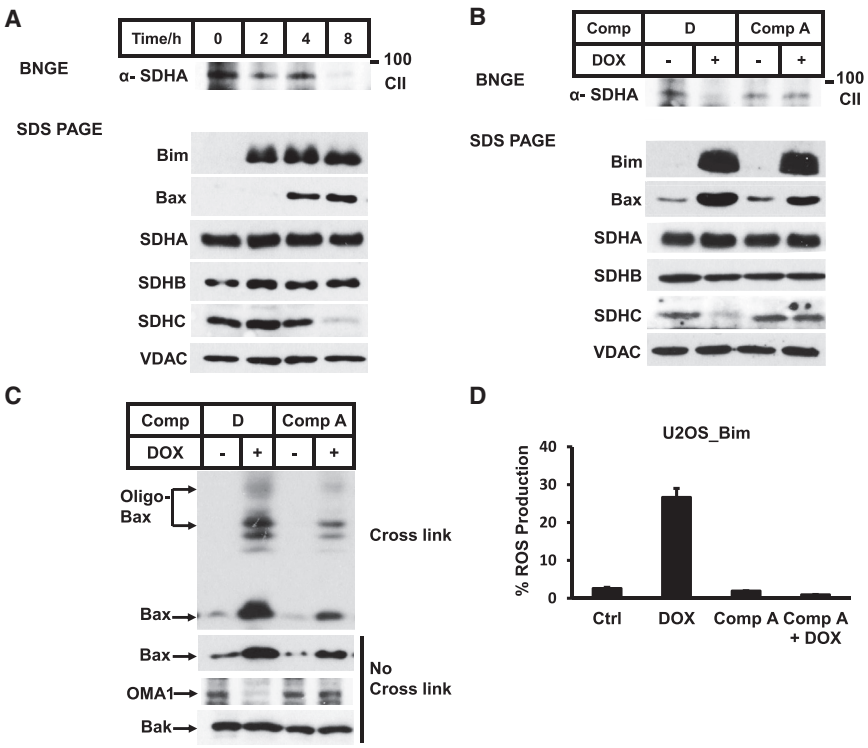

Figure 4. Compound A Preserved the Integrity of ETC Complex II during Apoptosis

(A) U2OS_Bim cells were treated with DOX for the times indicated. Lysates of the P15 fractions of the cells were analyzed by western blotting after blue native PAGE (BNGE) using anti-SDHA antibody (for detecting ETC complex II, CII), or SDS-PAGE using anti-Bim, anti-Bax, anti-SDHA, anti-SDHB, anti-SDHC, and anti-VDAC antibodies.

(B) U2OS_Bim cells were treated with DMSO or compound A for 1 hr and were then treated with or without DOX for 8 hr. Lysates of the P15 fractions of the cells were analyzed by western blotting after blue native PAGE using anti-SDHA antibody (for detecting CII) or SDS-PAGE using anti-Bim, anti-Bax, anti-SDHA, anti-SDHB, anti-SDHC, and anti-VDAC antibodies.

(C) U2OS_Bim cells were treated with DMSO or compound A for 1 hr and were then treated with or without DOX for 8 hr. The P15 fractions were cross-linked with 10 mM BMH where indicated and analyzed by western blotting using anti-Bax, anti-OMA1, and anti-Bak antibodies.

(D) U2OS_Bim cells were treated with DMSO or compound A for 1 hr and were then treated with or without DOX for 6 hr. Cells were then stained with 2′,7′-dichlorofluorescin diacetate (DCFDA), followed by flow cytometry analysis for detecting ROS production. Data are represented as the mean ± SD of duplicates. Ctrl, control.

SDHC and the disassembly of complex II were caused by the activity of Bax and Bak.

OMA1 is activated and cleaves OPA1 under a variety of mitochondrial stress conditions (Ehses et al., 2009; Head et al., 2009). OMA1 is also activated by the induction of Bim (Jiang et al., 2014). We therefore tested whether knockdown of OMA1 would affect SDHC degradation. Interestingly, knockdown of OMA1 did not affect SDHC degradation following Bim induction, indicating that degradation of SDHC is an upstream event relative to OMA1 activation (Figure S4D). Consistent with findings from a previous report (Jiang et al., 2014), knockdown of OMA1 attenuated OPA1 cleavage as well as cytochrome *c* release from the mitochondria and downstream caspase-9 activation (Figures S4D and S4E). Additionally, in OMA1 knockdown cells, the residual cell death was blocked by compound A (Figure S4F).

An Intact ETC Is Critical for Compound A to Protect Cells from Apoptosis

Our experiments up to this point clearly indicated that compound A blocks apoptosis by protecting the integrity of the ETC, which would otherwise be compromised following Bax/Bak activation. The disruption of the ETC would generate a burst of reactive oxygen species (ROS), which could potentially activate OMA1, leading to downstream apoptotic events. Indeed, induction of Bim in U2OS_Bim cells caused robust ROS generation that was blocked by compound A (Figure 4D). If ROS generation is downstream of a step where compound A exerts its function, then it is conceivable that compound A can be more efficacious when antioxidants are present. Indeed, both in cells treated with butylated hydroxyanisol (BHA) and in cells treated with Trolox, compound A showed improved cell protection activity (Figure S5A).

To further validate the supposition that the integrity of the ETC is important for the apoptosis-protecting effect of compound A, we generated U2OS_Bim ρ0 Cells with a defective ETC by introducing a DNAase into the mitochondrial matrix, as described previously (Saffran et al., 2007). The cells expressing the mitochondrial DNAase lost their mitochondrial genome, as detected by PCR amplification of a fragment within the D-loop region of mitochondrial DNA (Figure 5A), and, as a result, the mitochondrion- encoded protein COXII was not present in these cells (Figure 5B). The level of UQCRC1, a nucleus-encoded subunit of complex III, was also reduced to a non-detectable level in these cells. It seems plausible that UQCRC1 stability was reduced because other subunits of ETC complex III are mitochondrion-encoded. To the contrary, the level of SDHB, which is nucleus-encoded and is a component of ETC complex II that does not contain any mitochondrion-encoded subunits, was maintained at the same level as that in parental U2OS_Bim cells. Several other nucleus-encoded proteins that are not part of ETC complexes, including cytochrome *c* and VDAC, were maintained at normal levels (Figures 5B and 5C). As expected, both complex II and complex III of the ETC were disrupted in ρ0 cells (Figure 5C).

U2OS-Bim ρ0 cells still underwent apoptosis when Bim was induced by DOX (Figure 5D). Although compound A efficiently blocked apoptosis in parental U2OS_Bim cells, its efficacy decreased dramatically in ρ0 cells (Figure 5D). ρ0 cells thus behave like cells that have OPA1 defects. Indeed, cells that have had their endogenous OPA1 knocked down were also

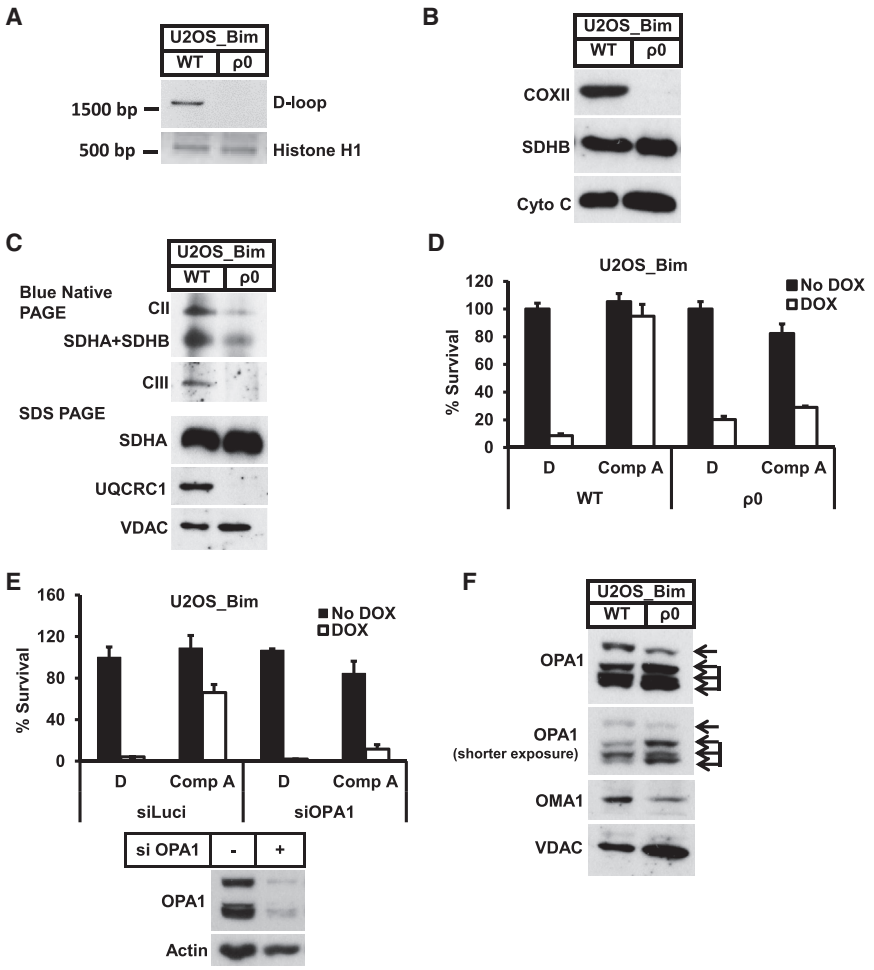

Figure 5. Depletion of Mitochondrial DNA Diminished the Effect of Compound A

(A) U2OS_Bim ρ0 cells were generated as described in the Experimental Procedures. DNA fragments within the D-loop region and the histone H1 gene of the WT and ρ0 U2OS_Bim cells were amplified via PCR.

(B) Lysates of the P15 fractions of the WT and ρ0 U2OS_Bim cells were analyzed by western blotting using anti-COX II, anti-SDHB, and anti-cytochrome *c* antibodies.

(C) Lysates of the P15 fractions of U2OS_Bim WT and ρ0 cells were analyzed by western blotting after blue native PAGE using anti-SDHA (for detecting CII and the SDHA/SDHB sub-complex) and anti-UQCRC1 (for detecting CIII) antibodies or SDS-PAGE using anti-SDHA, anti-UQCRC1, and anti-VDAC antibodies.

(D) U2OS_Bim WT and ρ0 cells were treated with DMSO (D) or compound A for 1 hr and were then treated with or without DOX for 24 hr. Cell survival was determined using a Cell Titer-Glo kit. Data are represented as the mean ± SD of duplicates.

(E) U2OS_Bim cells were transfected with siRNA against luciferase (Luci) or OPA1. After 48 hr, cells were treated with DMSO or compound A for 1 hr and were then treated with or without DOX for 24 hr. Cell viability was determined using a Cell Titer-Glo kit. Data are represented as the mean ± SD of duplicates. Whole-cell extracts of U2OS_Bim cells with or without OPA1 siRNA transfection were analyzed by western blotting using anti-OPA1 and anti-β-Actin-HRP antibodies.

(F) Lysates of the P15 fractions of the WT and ρ0 U2OS_Bim cells were analyzed by western blotting using anti-OPA1, anti-OMA1, and anti-VDAC antibodies.

no longer responsive to compound A (Figure 5E). Consistently, when the OPA1 protein was examined in ρ0 cells, it was obvious that some portion of the long-form OPA1 had already been cleaved and that the protease OMA1 had been activated; its level was decreased because of auto-cleavage (Figure 5F).

Consistent with the notion that an intact ETC is important for compound A to exert its cell protection function, when U2OS-Bim cells were treated with a chemical inhibitor of complex III of the ETC, antimycin A, the cell protection activity of compound A was obliterated (Figure S5B).

Compound A Saves Cells from Apoptotic Insults

Despite intensive efforts, researchers have as yet failed to identify a small molecule that can confer long-term survivability to cells when the mitochondrial apoptosis pathway is activated. The pan-caspase inhibitor z-VAD-FMK is able to keep cells alive (as measured by cellular ATP levels) during a short time frame (usually less than 24 hr) but is not able to save cells from death when the upstream insults to mitochondria persist.

We therefore tested the long-term effect of compound A on cells experiencing constant apoptotic insult. As shown in Figure 6A, control U2OS_Bim cells completely died off within 24 hr after the addition of DOX, and although the presence of z-VAD-FMK clearly delayed death, most of the cells had died by the third day. Remarkably, in the presence of compound A, the cells continued to proliferate, similar to cells grown under normal conditions.

The long-term cell survivability conferred by compound A was again demonstrated when the cells were cultured for 1 week and the resulting cell colonies were visualized with crystal violet dye (Figure 6B). The U2OS_Bim cells were all killed when DOX was added, even in the presence of z-VAD-FMK. In contrast, when compound A was present, the cell colonies grown in the medium containing DOX were indistinguishable from the ones grown without DOX, demonstrating that compound A, unlike the caspase inhibitors, which only delay cell death, is able to keep the cells alive and proliferating even after their mitochondrial apoptotic pathway has been activated.

Compound A Is Effective in Protecting Neuronal Death in an Animal Model of Parkinson's Disease

The remarkable cell protection effect of compound A that we observed with our in vitro experiments prompted us to test whether the compound might show similar effects in in vivo experiments. For this purpose, we chose a 6-hydroxydopamine (6-OHDA)-induced Parkinson's disease model in rats.

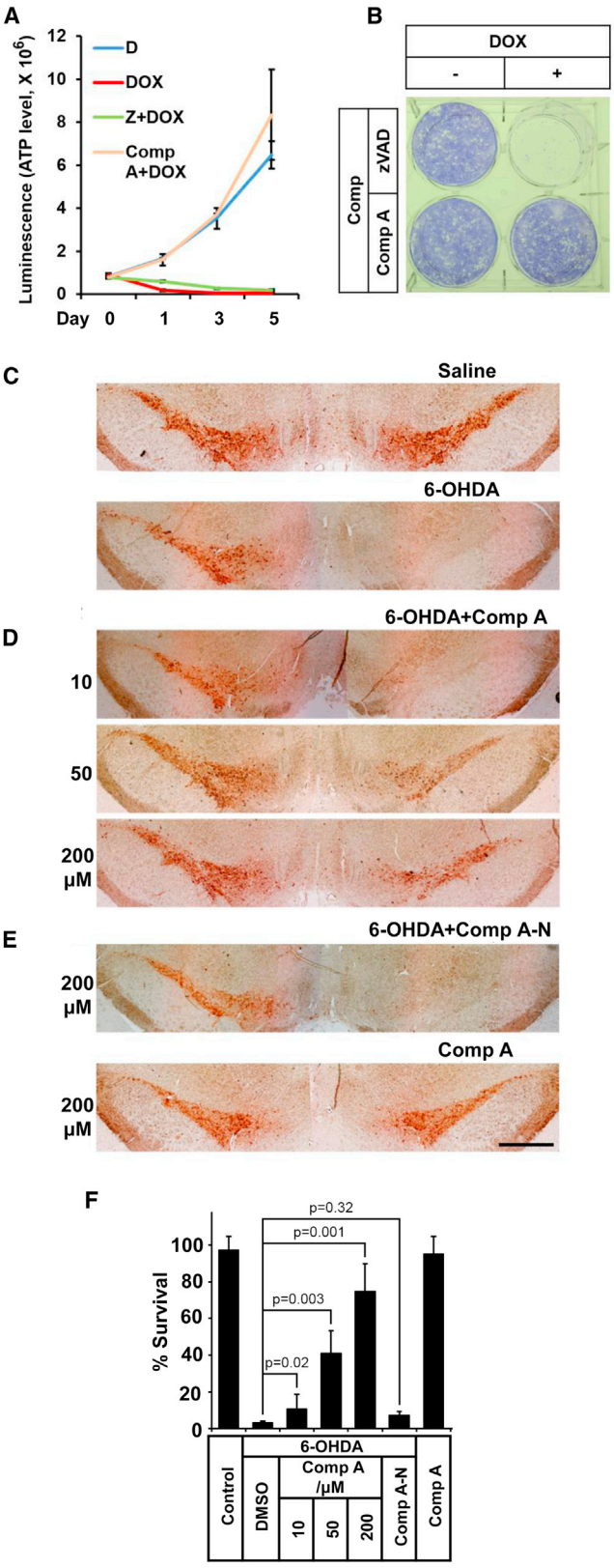

Figure 6. Compound A Sustained Cell Growth after the Induction of Apoptosis and Prevented 6-OHDA-Induced Neurotoxicity

(A and B) U2OS_Bim cells were seeded in 96-well plates at a density of 500 cells/well. On the next day, cells were treated with the indicated compounds for 1 hr and were then treated with or without DOX. Cell survival was determined by measuring ATP levels using a Cell Titer-Glo kit immediately after DOX treatment (day 0) or 1, 3, or 5 days later (days 1, 3, 5) (A). Data are represented as the mean ± SD of duplicates. Colony formation was analyzed using crystal violet staining as described in the Experimental Procedures (B).

(C) Representative montage photomicrograph showing the effect of 6-OHDA on tyrosine hydroxylase (TH) immunostaining in the rat substantia nigra (SN) 14 days after injection. In a saline-injected control, there were dense TH-positive cell bodies in the SN. After the unilateral injection of 6-OHDA, the TH immunoreactivity was significantly reduced at the injection site.

(D and E) Compound A (D) or a negative compound A analog, Comp A-N (E), was injected with 6-OHDA at the indicated concentrations. Compound A was also injected in the absence of 6-OHDA (E). TH immunoreactivity was analyzed 14 days after injection. Scale bar, 1 mm.

(F) Survival rate (relative to the uninjected site) of neurons in the SN as assessed by optical density. Each group contained three to six rats, and three sections from each rat were analyzed. Differences between means were assessed by one-way ANOVA with post hoc Tukey's analysis, and p values are shown. Error bars represent the mean ± SD.

The injection of the dopamine derivative 6-OHDA into the medial forebrain bundle region of the rat brain caused specific depletion of dopaminergic neurons in the substantia nigra region (Figure 6C, bottom). Co-injection of increasing amounts of compound A showed dose-dependent protection of these neurons (Figures 6D and 6F). The inactive derivative of compound A, even at the highest injected concentration, did not show any protective effect (Figures S6A, S6B, and S6E). Treatment with compound A alone did not increase the area of the substantia nigra region, indicating that the effect of compound A occurred via cell protection and not from increased cell proliferation. The protective effect of compound A did not result from a chemical reaction that neutralized 6-OHDA because similar amounts of 6-OHDA were detected in rat brain tissue regardless of the presence or absence of compound A (Figures S6C–S6F). The dopaminergic neurons protected by compound A appeared to be functional because the Parkinson-like behavior of 6-OHDA-injected rats was corrected by compound A (Movies S1, S2, and S3).

DISCUSSION

Disruption of the Mitochondrial Electron Transfer Chain Is Required for the Execution of the Mitochondrial Apoptotic Pathway

The finding that compound A blocks apoptosis after Bax/Bak activation by targeting a component of the ETC suggests that the activated Bax and Bak are able to affect the mitochondrial ETC in addition to making protein-permeable pores on the outer membrane. The disruption of the ETC leads to the generation of ROS and then activation of OMA1, which subsequently cleaves the long-form OPA1. The cleavage of OPA1 causes disassembly of the OPA-1-containing complex and opens up the cristae, allowing the majority of cytochrome *c* to gain access to the outer membrane. This two-step process of cytochrome *c* release, namely the remodeling of cristae caused by disruption of the

ETC and permeabilization of the outer membrane, can now be clearly demonstrated with compound A. Compound A only blocks the disruption of the ETC without altering the permeability of the outer mitochondrial membrane. Thus, in ρ0 cells, because the ETC is already disrupted, these cells appear to undergo apoptosis normally, and apoptosis can still be blocked by Bcl-2 (Jacobson et al., 1993); however, compound A is no longer effective in blocking apoptosis in these cells (Figure 5). We hypothesize that the degree of ETC integrity might dictate how effective compound A is and, furthermore, may account for the divergent efficacy of compound A in blocking apoptosis, which was observed in different cell lines treated with various apoptotic stimulations (Figures S1C and S1D).

Mutations in the *SDHB*, *SDHC*, and *SDHD* genes have been reported to cause instances of rare cancers such as familial pheochromocytoma and familial paraganglioma (Astuti et al., 2001). Moreover, disruption of complex II of the ETC was observed when cells were induced to undergo apoptosis via treatment with several chemical reagents, including a calcium ionophore (Hwang et al., 2014). ETC complex II might be a common target for a diverse battery of apoptotic insults.

The Proposed Mechanism of How Compound A Blocks the Disruption of ETC Complex II

The disruption of ETC complex II depends on the activity of Bax/Bak (Figure S5C) and occurs after Bax translocation to the mitochondria (Figure 4B). Compound A completely blocks ETC complex II disruption but only modestly affects Bax oligomerization (Figures 4B and 4C). Therefore, compound A most likely functions downstream of Bax/Bak activation. The direct target of compound A is the SDHB of complex II, and the causal biochemical alteration is the covalent modification of cysteine 243. Despite the fact that cysteine 243 is one of the three cysteine residues whose side-chain thiolates serve as ligands for the [3Fe-4S] cluster, neither the modification of compound A on cysteine 243 nor mutation of this amino acid to an alanine residue affected its normal electron transfer activity (Figures 1D and 3E).

Both SDHC and SDHD have three transmembrane helixes that anchor ETC complex II to the inner membrane of mitochondria. SDHA and SDHB, the subunits facing the mitochondrial matrix, are connected to the membrane through hydrophobic interactions between the first N-terminal α helix plus the connecting loop region of SDHC and the C-terminal part of SDHB, where cysteine 243 resides (Sun et al., 2005). It is thus conceivable that, after the methylsulfonyl moiety of compound A is linked to cysteine 243, the rest of the structural features of compound A, including the benzyl ring, the trifluoromethylated pyrimidin ring, and the pyridine ring, may provide additional hydrophobic interactions between SDHB and SDHC. Such additional interactions between SDHB and SDHC may directly stabilize ETC complex II. Alternatively, because SDHC is degraded during apoptosis, and this degradation should result in the disassembly of complex II and disassociation of the SDHA/SDHB sub-complex from the membrane, compound A may prevent the disruption of ETC complex II by inhibiting the degradation of SDHC.

The latter scenario predicts the existence of a mitochondrial protease (or proteases) that is activated by oligomer Bax/Bak. This putative protease(s) would degrade SDHC, leading to the observed disassembly of ETC complex II. Cysteine 243-compound A of SDHB inhibits the degradation of SDHC by virtue of being close to the putative cleavage site.

The protease that degrades SDHC does not seem to be OMA1, a mitochondrial inner membrane protease known to be activated during apoptosis at a point downstream of Bax/Bak activation (Jiang et al., 2014). Knockdown of OMA1 did not prevent the cleavage of SDHC, although it did partially block both cytochrome *c* release from mitochondria and downstream caspase activation (Figures S4D and S4E).

The Point of No Return for Mitochondrion-Mediated Apoptosis

There has been a longstanding debate about which point of the apoptosis pathway can be termed the point of no return; i.e., the point beyond which cells are unable to recover and invariably die. Genetic studies of *C. elegans* suggested that the somatic cells destined to die were all saved if any of the components of the apoptotic pathway, including the caspase Ced-3, were defective (Ellis and Horvitz, 1986). However, in mammalian cells, caspase inhibition is not able to save cells that are experiencing apoptotic insults that target mitochondria. Our present study indicates that, if the disruption of ETC complex II and the disruption of the complex II-containing supercomplex are prevented by compound A, then the cells' long-term survival is preserved even though Bax and Bak are still oligomerized and even though the outer membrane of mitochondria has presumably already become permeable. The disruption of ETC complex II, therefore, fulfills the criteria for the point of no return in the mitochondrial apoptotic pathway.

Compound A Promotes Cell Survival in Degenerative Disease Models

Mitochondrial damage results from a variety of genetic and environmental stresses, and aging has been considered to be a major factor in the development of neurodegenerative diseases (reviewed by Lin and Beal, 2006). Such damage can cause both apoptotic and non-apoptotic cell death, depending on the execution of downstream caspase activation pathways, but is detrimental to neurons regardless. These cell death events can potentially be prevented by therapies based on compound A.

For example, in a Parkinson's disease model induced by the dopamine derivative 6-OHDA, compound A conferred dose-dependent protection of dopaminergic neurons and corrected the neurological phenotype associated with the disease. This compound can now be used in a variety of degenerative diseases models to evaluate potential therapeutic effects.

EXPERIMENTAL PROCEDURES

Reagents

General chemicals were purchased from Sigma unless otherwise stated. Bismaleimidohexane (BMH) and streptavidin agarose were purchased from Life Technologies. zVAD was obtained from WuXi AppTec. Compound A and its analogs were synthesized in the lab of Z.Z. at the National Institute of Biological Sciences. The Cell Titer-Glo kit was purchased from Promega. The following antibodies were used: anti-Bim (2819, Cell Signaling Technology), anti-caspase 9 (9502, Cell Signaling Technology), anti-caspase 3 (9662, Cell Signaling Technology), anti-β-Actin-horseradish peroxidase (HRP) (D291-7,

MBL), anti-cytochrome *c* (556433, BD Pharmingen), anti-Bax (5023, Cell Signaling Technology), anti-Bak (3814, Cell Signaling Technology), anti-VDAC (4866, Cell Signaling Technology), anti-OPA1 (612606, BD Biosciences), anti-OMA1 (sc-168844, Santa Cruz Biotechnology), anti-FLAG-HRP (A2859, Sigma), anti-SDHA (ab14715, Abcam), anti-SDHB (sc-25851, Santa Cruz Biotechnology), anti-SDHC (ab125411, Abcam), anti-streptavidin-HRP (3999S, Cell Signaling Technology), anti-UQCRC1 (sc-367801, Santa Cruz Biotechnology), and anti-COX II (55070-1-AP, Proteintech).

Plasmids and siRNA Oligos

The FLAG-tagged wild-type and C243A mutant SDHB plasmids were constructed in a modified pcDNA 3.1 plasmid with a 3× FLAG tag at the C-terminal end. The SDHB guide RNA (gRNA) plasmid was constructed in a modified gRNA-cloning vector (Addgene) containing a Cas9 transgene. The gRNA target sequence was GTCCTTTCAGTGTGAGCCTA. The targeting vector was constructed in a modified pEasy Flox vector (Addgene) as described in the Supplemental Experimental Procedures. The sequence of the OPA1 small interfering RNA (siRNA) was AACGGCGUUUAGAGCAACAGA.

Transfection and Stable Cell Lines

Transfection of the cells using Lipofectamine 2000 (Invitrogen) was performed according to the manufacturer's instructions. The U2OS_Bim cell line was established previously (Jiang et al., 2014). To generate the SDHB C243A knockin cell line, U2OS_Bim cells were infected with lentiviral particles containing the SDHB gRNA plasmid followed by transfection with the targeting vector. Cells were then selected with 500 μg/ml hygromycin (Roche). The genomic DNA of the surviving clones was analyzed via PCR using a pair of knockin allele-specific primers. The 30761-31760 region of the SDHB genomic DNA sequence of the PCR-positive clones was amplified via PCR and analyzed by BamHI digestion and sequencing, finally enabling the identification of the SDHB C243A knockin clones. The primers used for generating the knockin cell line are detailed in the Supplemental Experimental Procedures.

Cell Viability Assay

Cell viability was analyzed by measuring ATP levels using a Cell Titer-Glo kit according to the manufacturer's instructions.

Immunoprecipitation

P15 fractions of the cells were lysed in lysis buffer (50 mM Tris [pH 8.0], 137 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, and 1× protease inhibitor mixture [Roche]) on ice for 30 min and then centrifuged at 18,000 × *g* for 20 min at 4°C. The supernatants were saved as the input materials for immunoprecipitation using streptavidin agarose beads. The streptavidin agarose beads were pre-coated with the biotinylated compounds (20 μM) for 2 hr at room temperature (RT) and were then incubated with the P15 lysates for 2 hr at RT. For the competition experiments, the lysates were pre-incubated with 1 μM compound A for 2 hr at RT. Silver staining was carried out using a silver staining kit (Sigma, LC6070) according to the manufacturer's instructions. The protein bands of interest were then cut and analyzed by mass spectrometry.

Blue Native PAGE

Lysates of the P15 fractions of the cells were prepared with 0.25% G250 (BN2004, Life Technologies) and a 1/4 volume of 4× native sample buffer (BN2003, Life Technologies). 10 μg of protein for each sample was loaded on 3%–12% Bis-Tris native gels (BN1001BOX), and gel electrophoresis was performed according to the manufacturer's instructions.

Generation of ρ0 Cells

The ρ0 cells were established by expressing an amino-terminal truncated version of the herpes simplex virus protein UL12 (starting from codon 127) (Saffran et al., 2007) in parental cells, as described in the Supplemental Experimental Procedures.

Full experimental procedures for the cellular fractionation, cross-linking assays, oxygen consumption rate measurement, cell growth assay, crystal violet staining, 6-OHDA lesions, compound administration, and immunohistochemistry are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2016.06.016.

AUTHOR CONTRIBUTIONS

X.J. and X.W. designed the research and analyzed the data. X.J. characterized compound A and identified its target. Li Li, S.H. and Z.Z. helped develop and synthesize compound A. Z.Y. and C.P. performed the animal experiment. Lin Li and S.C. did the mass spectrometry analysis. M.D. helped generate the knockin cell line. B.Y. performed the compound stability analysis. M.L. did the EM experiment. H.J. helped carry out the compound screening. X.W. wrote the manuscript.

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